Supplementary Material

RAS isoform specific activities are disrupted by disease associated mutations during cell differentiation

Rohan Chippalkatti[1a], Bianca Parisi[1a], Farah Kouzi[1], Christina Laurini[1], Nesrine Ben Fredj[1] and Daniel Kwaku Abankwa[1*]

[1]Cancer Cell Biology and Drug Discovery group, Department of Life Sciences and Medicine, University of Luxembourg, 4362, Esch-sur-Alzette, Luxembourg

[a] these authors contributed equally

[*]correspondence: daniel.abankwa@uni.lu

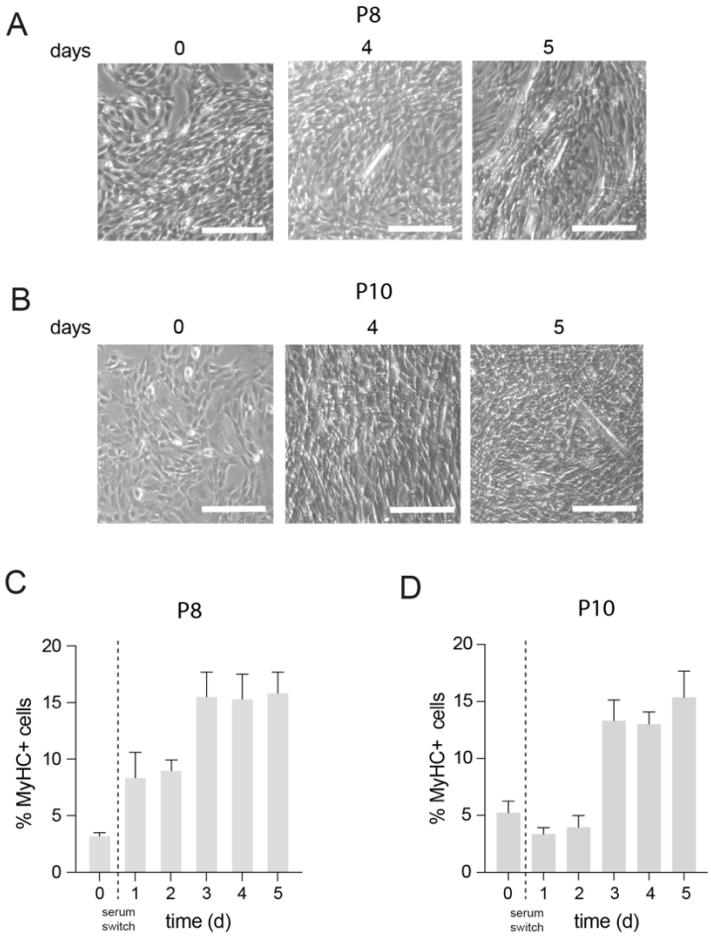

Fig. S1. Cell batches with a high passage number show reduced differentiation potential.

(**A,B**) Brightfield images (20 ×) of C2C12 cells with passage numbers (P) P8 (A) and P10 (B) at indicated times in low serum medium. Scale bars, 200 µm. (**C,D**) Flow cytometric quantification of MyHC+ C2C12 cells with passage number 8 (C) and 10 (D) at indicated times of differentiation; $N \geq 2$. Higher passage numbers appear to increase the MyHC+ fraction on day 0 and show a decreased differentiation potential subsequently.

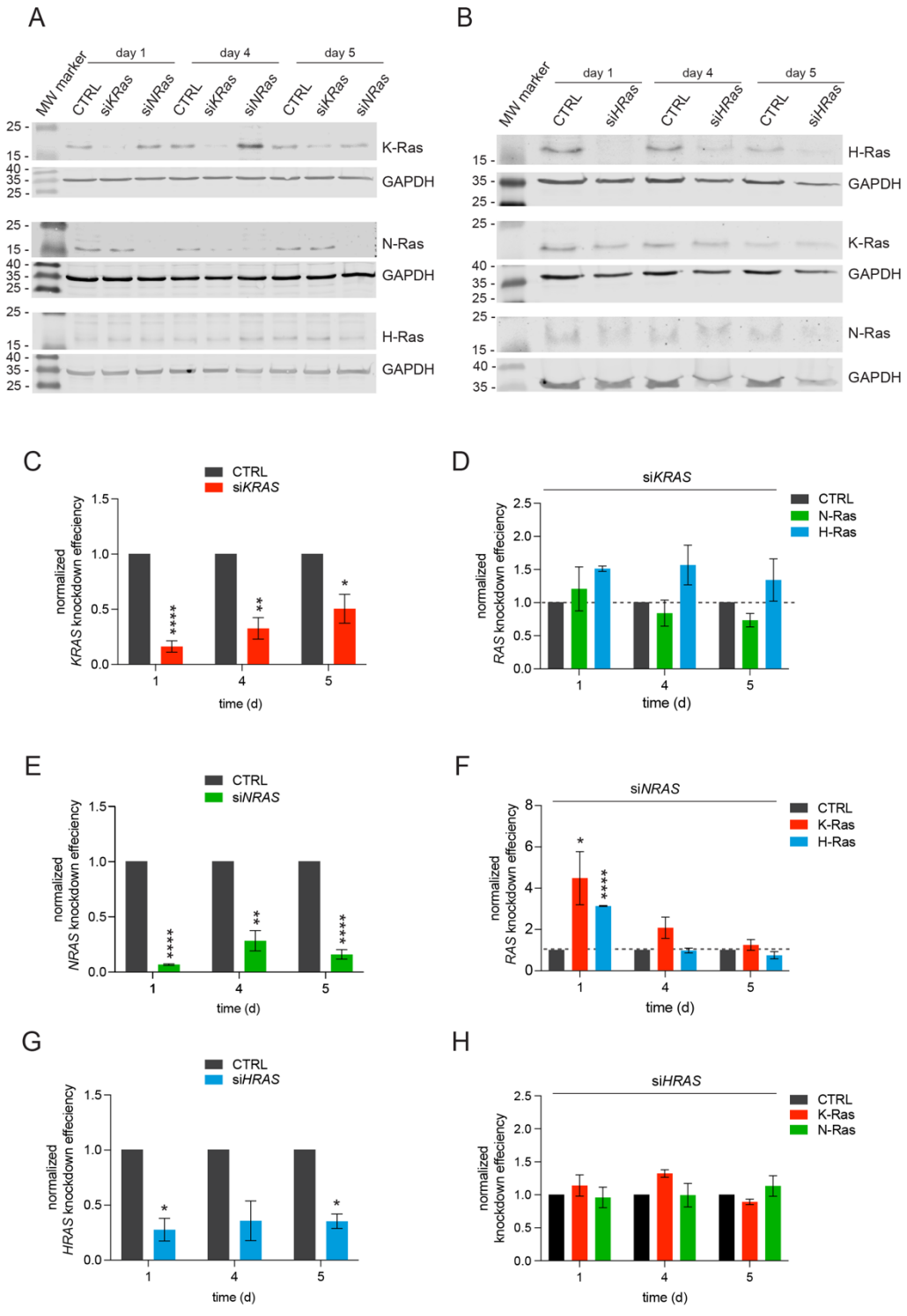

Fig. S2. Protein level quantification after knockdown of endogenous *KRAS*, *NRAS* and *HRAS* using isoform-specific siRNAs.

(**A,B**) Representative immunoblots of lysates from C2C12 cells with *KRAS* and *NRAS* (A) and *HRAS* (B) knockdowns on day 0. Cells were differentiated for indicated times in low serum medium; N = 3. Note that the K-Ras antibody may detect preferentially K-Ras4B (**C,D**) Quantification of data as in (A,B) for *KRAS* knockdown (C) and effect of *KRAS* siRNA

on protein levels of N-Ras and H-Ras to assess specificity (D); N = 3. (**E-H**) Analogous analysis as in (C,D) was performed for *NRAS* knockdowns (E,F) and *HRAS* knockdowns (G,H); N = 3.

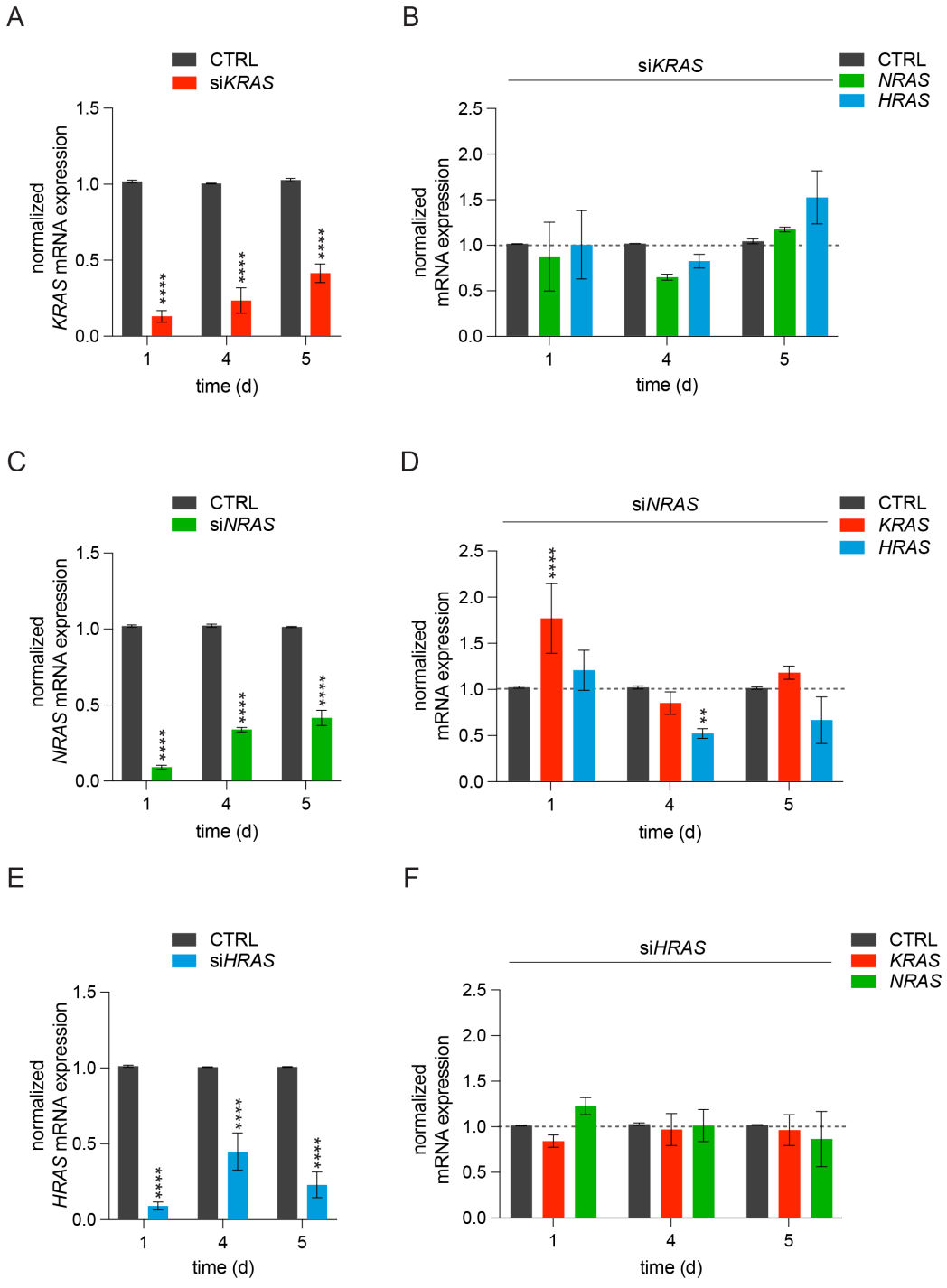

Fig. S3. Quantification of mRNA-levels after knockdown of endogenous *KRAS*, *NRAS* and *HRAS* using isoform-specific siRNAs.

(**A,B**) Quantitative RT-PCR was employed to validate knockdown of indicated *RAS*-transcripts after si*KRAS* treatment of C2C12 cells on day 0. Cells were differentiated for indicated times in low serum medium (A). Analysis of the specificity by quantitating *NRAS*- and *HRAS*- transcripts after the same treatment (B); N = 3, passage number 6-7. Expression

levels relative to GAPDH were normalized to samples treated with CTRL siRNA per time point. (**C-F**) Analogous analysis as in (A,B) was performed for *NRAS* knockdowns (C,D) and *HRAS* knockdowns (E,F); N = 3.

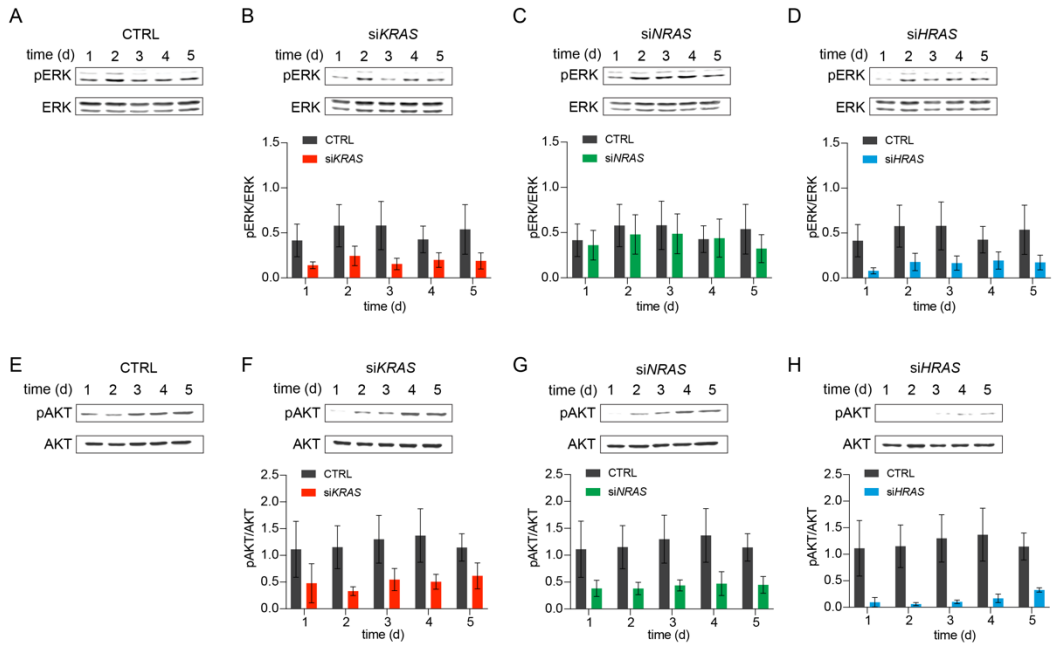

Fig. S4. Quantification of relative pERK1/2- and pAKT-levels after knockdown of endogenous *KRAS*, *NRAS* and *HRAS* using isoform-specific siRNAs.

(**A-D**) Representative immunoblots of C2C12 cell lysates (top) and their quantified normalized pERK signals after day 0 knockdown with CTRL siRNA (A) or siRNAs against *KRAS* (B), *NRAS* (C) and *HRAS* (D); N = 3, passage number 6-7. (**D-F**) Representative immunoblots of C2C12 cell lysates (top) and their quantified normalized pAkt signals after day 0 knockdown with CTRL siRNA (A) or siRNAs against *KRAS* (B), *NRAS* (C) and *HRAS* (D); N = 3, passage number 6-7.

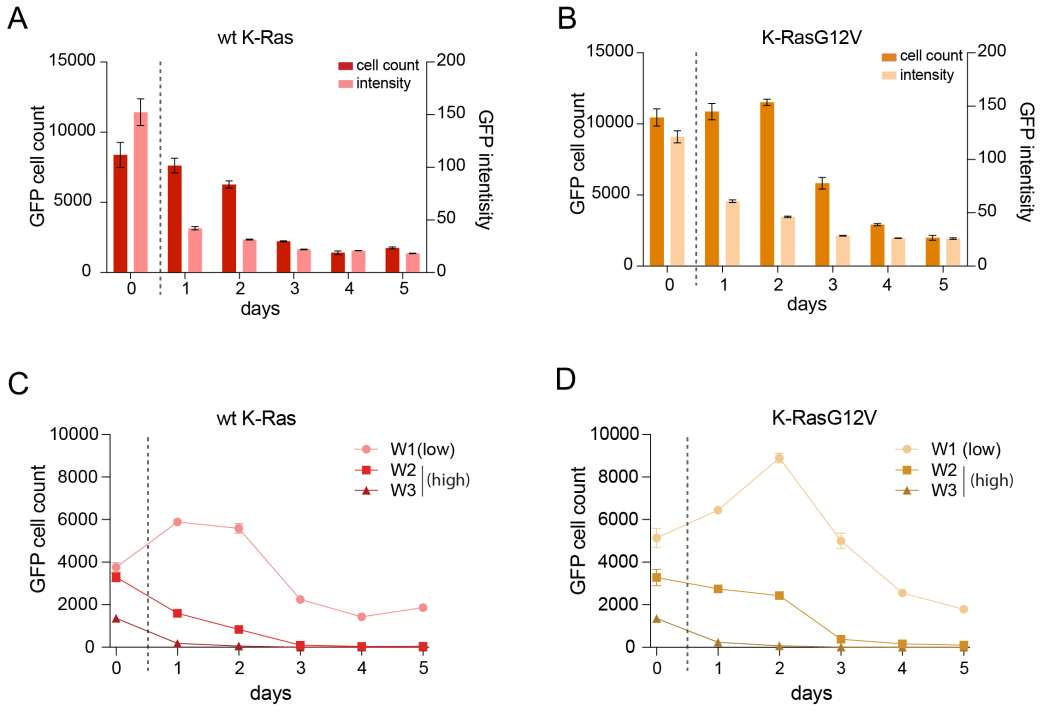

Fig. S5. Analysis of 'low' and 'high' GFP-expression windows in cells transfected with mEGFP-wt K-Ras and mEGFP-K-RasG12V.

(**A,B**) Flow cytometric analysis of GFP+ fraction of C2C12 cells in comparison to geometric means of intensities for mEGFP-wt K-Ras (A) and mEGFP-K-RasG12V (B) expressing cells; N = 3, passage number 6. Data suggest that differentiation was perturbed by exogenously expressed Ras-constructs mainly on days 1 and 2. (**C,D**) Flow cytometric analysis of GFP+ fraction of C2C12 cells in 3 distinct GFP-construct expression windows for mEGFP-wt K-Ras (A) and mEGFP-K-RasG12V (B) expressing cells; N = 3. Relative expression window range (W) with fold signal over background of autofluorescence of non-labelled cells, W1: 10^1 to 10^2 RFU (corresponds to GFP low); W2: 10^2 to 10^3 RFU; W3: 10^3 to 10^4 RFU; W2 + W3 correspond to GFP high.

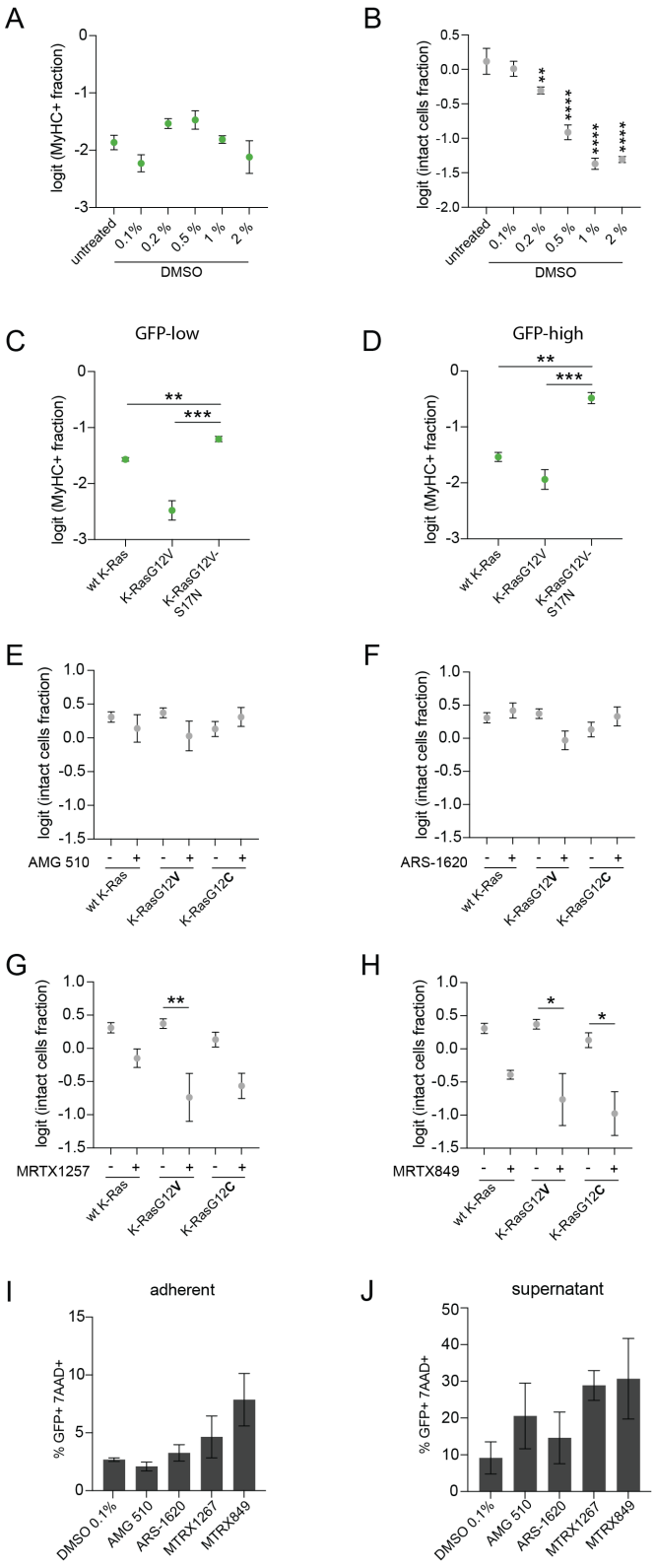

Fig. S6. RAS-controls and assessment of cell toxicity in C2C12 cells.
(**A**) Flow cytometric analysis of MyHC+ C2C12 cells treated with indicated concentrations of DMSO vehicle during the 3-day differentiation; N = 3, passage number 8. (**B**) Flow

cytometric quantification of intact cells based on forward- and side-scatter in samples from (A) indicates treatment induced toxicity if this fraction decreases. (**C,D**) Flow cytometric analysis of MyHC+ C2C12 cell fractions expressing EGFP-variant tagged wt K-Ras, K-RasG12V or K-RasG12V-S17N on day 3 of differentiation; N = 3, passage number 8. Cells were analyzed in the GFP low (C) and GFP high (D) windows, after transfection on day 0. (**E-H**) Analysis of intact cell fractions determined by flow cytometry based on forward- and side-scatter properties of samples as in Figure 6 after treatments with 3 μM AMG 510 (E), ARS-1620 (F), MRTX1257 (G) and MRTX849 (H) expressed by; N = 4, passage number 9. Lower values indicate compound-induced toxicity if this fraction decreases. (**I,J**) Flow cytometric quantification of GFP+ 7AAD+ late apoptotic/ necrotic C2C12 cells transfected on day 0 with mEGFP-K-RasG12C and with indicated treatments during 3-days of differentiation; N = 3. Cells adherent (I) or in the supernatant (J) were analyzed separately.

Table S1. Key resources table. List of materials and reagents used in this study.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Pax7 (Pax7); dilution 1:100 flow cytometry (FC)	Bio-Techne	Cat# MAB1675 RRID: AB_2159833
Mouse monoclonal anti-myogenin (5FD); dilution 1:100 FC; dilution 1:500 Western blotting (WB)	ThermoFisher Scientific	Cat# 14-5643-82 RRID: AB_1907431
Mouse monoclonal anti-Myosin 4 (MF20), eFluor 660; dilution 1:100 FC	ThermoFisher Scientific	Cat# 50-6503-82 RRID: AB_2574267
Mouse monoclonal anti-Myosin 4 (MF20); dilution 1:100 FC	ThermoFisher Scientific	Cat# 14-6503-82 RRID: AB_2572894
Mouse monoclonal anti-Pax7, Alexa Fluor 488 (Pax7); dilution 1:25 FC	Santa Cruz Biotechnology	Cat# sc81648 RRID: AB_2159836
Mouse monoclonal anti-Histone H3 phospho (Ser10), Alexa fluor 647 (11D8); dilution 1:20 FC	BioLegend	Cat# 650805 RRID: AB_2564361
Mouse monoclonal anti-GAPDH, (GAPDH-71.1); dilution 1:10,000 WB	Merck	Cat# G8795 RRID: AB_1078991
Rabbit polyclonal anti-GAPDH; dilution 1:10,000 WB	Merck	Cat# G9545 RRID: AB_796208
Mouse monoclonal anti-Pax7 (Pax7); dilution 1:500 WB	Santa Cruz Biotechnology	Cat# sc-81648 RRID: AB_2159836
Rabbit polyclonal anti-MYH1 (N-terminal); dilution 1:500 WB	Proteintech	Cat# 25182-1-AP RRID: AB_2879947
Rabbit polyclonal anti-p44/42 MAPK (ERK1/2); dilution 1:1000 WB	Cell Signaling Technology	Cat# 9102 RRID: AB_330744
Mouse monoclonal anti-phopsho-p44/42 MAPK (ERK1/2) (Thr202/Tyr 204) (E10); dilution 1:2000 WB	Cell Signaling Technology	Cat# 9106 RRID: AB_331768
Mouse monoclonal anti-Akt (pan) (40D4); dilution 1:2000 WB	Cell Signaling Technology	Cat# 2920 RRID: AB_1147620
Rabbit monoclonal anti-phospho-Akt (Ser473) (D9E); dilution 1:1000 WB	Cell Signaling Technology	Cat# 4060 RRID: AB_2315049
Mouse monoclonal anti-KRAS (3B10-F2); dilution 1:1000 WB	Merck	Cat# WH0003845M1 RRID: AB_1842235
Rabbit polyclonal anti-HRAS; dilution 1:1000 WB	Proteintech	Cat# 18295-1-AP RRID: AB_2121046
Mouse monoclonal anti-NRAS (F155); dilution 1:1000 WB (dilution 1:250 for si*HRAS*)	Santa Cruz Biotechnology	Cat# sc-31 RRID: AB_628041

IRDye 680RD goat anti-Rabbit IgG; dilution 1:10,000 WB	LI-COR Biosciences	Cat# 926-68071 RRID: AB_10956166
IRDye 680LT donkey anti-Mouse IgG; dilution 1:10,000 WB	LI-COR Biosciences	Cat# 926-68022 RRID: AB_10715072
IRDye 800CW goat anti-Rabbit IgG; dilution 1:10,000 WB	LI-COR Biosciences	Cat# 926-32211 RRID: AB_621843
IRDye 800CW donkey anti-Mouse IgG; dilution 1:10,000 WB	LI-COR Biosciences	Cat# 926-32212 RRID: AB_621847
Bacterial and virus strains		
E. coli DH10B	New England BioLabs	Cat# C3019I
Biological samples		
N/A	N/A	N/A
Chemicals, peptides, and recombinant proteins		
Sotorasib (AMG 510)	MedChem Express	Cat# HY-114277 CAS# 2296729-00-3
ARS-1620	MedChem Express	Cat# HY-U00418 CAS# 1698055-85-4
MRTX1257	MedChem Express	Cat# HY-114436 CAS# 2206736-04-9
MRTX1133	MedChem Express	Cat# HY-134813 CAS# 2621928-55-8
Trametinib	MedChem Express	Cat# HY-10999 CAS# 871700-17-3
Adagrasib (MRTX849)	Selleckchem	Cat# S8884 CAS# 2326521-71-3
Cysmethynil	Cayman Chemical	Cat# 14745 CAS# 851636-83-4
Rapamycin	BioVision	Cat# 1568 CAS# 53123-88-9
Tipifarnib	Selleckchem	Cat# S1453 CAS# 192185-72-1
BI-3406	MedChem Express	Cat# HY-125817 CAS# 2230836-55-0
Gefitinib	Biorbyt	Cat# orb545838 CAS# 184475-35-2
Dimethyl sulfoxide (DMSO)	PanReac AppliChem	Cat# A3672 CAS# 67-68-5
Critical commercial assays		
Gateway LR Clonase II enzyme mix	ThermoFisher Scientific	Cat# 11791020
APC conjugation kit – Lightning-Link	Abcam	Cat# ab201807
PE/R-Phycoerythrin conjugation kit – Lightning Link	Abcam	Cat# ab102918
Cell proliferation staining reagent – Deep Red Fluorescence (Cytopainter deep red)	Abcam	Cat# ab176736

Cell proliferation staining reagent – Orange Fluorescence (Cytopainter orange)	Abcam	Cat# ab176737
Bio-Rad Protein Assay Kit I	Bio-Rad	Cat# 5000001
Pierce Protease Inhibitor Tablets	ThermoFisher Scientific	Cat# A32963
PhosSTOP	Merck	Cat# 4906845001
Lipofectamine RNAiMAX Transfection Reagent	ThermoFisher Scientific	Cat# 13778075
Lipofectamine 2000 Transfection Reagent	ThermoFisher Scientific	Cat# 11668019
TRIzol Reagent	ThermoFisher Scientific	Cat# 15596018
SuperScriptIII Reverse Transcriptase	ThermoFisher Scientific	Cat# 18080093
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad	Cat# 1725274
FITC Annexin V Apoptosis detection kit I	BD Biosciences	Cat# 556547
Experimental models: Cell lines		
Mouse cell line, C2C12	ATCC	Cat# CRL-1772 RRID: CVCL_6974
Experimental models: Organisms/strains		
N/A	N/A	N/A
Oligonucleotides		
Custom qPCR primer: KRAS human_F 5'-3' sequence TACAGTGCAATGAGGGACCA	Genecust	N/A
Custom qPCR primer: KRAS human_R 5'-3' sequence TCCTGAGCCTGTTTTGTGTCT	Genecust	N/A
Custom qPCR primer: NRAS mouse_F 5'-3'sequence GGACAGTTGACACAAAGCAAGCC	Origene/ IDT DNA	N/A
Custom qPCR primer: NRAS mouse_R 5'-3' sequence TGGCGTATCTCCCTTACCAGTG	Origene/ IDT DNA	N/A
Custom qPCR primer: HRAS mouse_F 5'-3' sequence AGCAGATCAAGCGGGTGAAA	ThermoFisher Scientific	N/A
Custom qPCR primer: HRAS mouse_R 5'-3' sequence ATCGGGTGGGTTCAGTTTCC	ThermoFisher Scientific	N/A
Custom qPCR primer: GAPDH mouse_F 5'-3' sequence TGGTGAAGGTCGGTGTGAA	Eurogentec	N/A
Custom qPCR primer: GAPDH mouse_R 5'-3' sequence ATGAAGGGGTCGTTGATGG	Eurogentec	N/A
Negative control siRNA	QIAGEN	Cat# 1027310

		GeneGlobe ID: SI03650325
Human *HRAS* siRNA (FlexiTube siRNA) 5'-3'sequence CGGAAGCAGGUGGUCAUUA	QIAGEN	Cat# 1027417 GeneGlobe ID: SI02654806
Mouse *KRAS* siRNA (targeting both *KRAS*4A and 4B transcripts) (ON-TARGETplus SMARTpool siRNA) 5'-3' sequences GAACAGUAGACACGAAAA AGCAAGGAGUUACGGGAUU GGUUGGAGCUGGUGGCGUA GGUGUACAGUUAUGUGAAU	Horizon Discovery	Cat# L-043846-01-0005
Custom siRNA: Mouse *NRAS* siRNA 5'-3' sequence GCAAAUUAAGCGUGUGAAAUUU	Horizon Discovery	N/A
Recombinant DNA		
C453-E04: attB4-CMV51p>-attB5	FNL Combinatorial Cloning Platform, RAS-Initiative	Addgene# 162973
pDest-305 (attR4-attR2)	FNL Combinatorial Cloning Platform, RAS-Initiative	Addgene# 161895
C512-E01: attB5r-mEGFP-nostop-attB1r	FNL Combinatorial Cloning Platform, RAS-Initiative	Addgene# 162940
Hs. KRAS4b-WT	RAS mutant collection V2.0, RAS-Initiative	Addgene# 83129
Hs. KRAS4b-G12C	RAS mutant collection V2.0, RAS-Initiative	Addgene# 83130
Hs. KRAS4b-G13D	RAS mutant collection V2.0, RAS-Initiative	Addgene# 83133
Hs. KRAS4b-Q61H	RAS mutant collection V2.0, RAS-Initiative	Addgene# 83140
pDest305-CMV-mEGFP-K-Ras4B WT	This paper	N/A
pDest305-CMV-mEGFP-K-Ras4B G12C	This paper	N/A
pDest305-CMV-mEGFP-K-Ras4B G13D	This paper	N/A
pDest305-CMV-mEGFP-K-Ras4B Q61H	This paper	N/A
pmEGFP-KRas4B-G12V	{Abankwa, 2010 #224}	N/A

pmEGFP-HRas-G12V	{Abankwa, 2010 #224}	N/A
pmEGFP-HA-NRas-G12V	{Abankwa, 2010 #224}	N/A
pEGFP-KRAS4B-G12D	{Yelland, 2022 #225}	N/A
pmEGFP-KRas4B-G12V-S17N	Gene Universal	N/A
pEYFP-wt KRas	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-T58I	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-D153V	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-V14I	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-P34R	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-P34L	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-K5N	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-K5E	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-Q22E	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-G60R	{Gremer, 2011 #226}	N/A
pEYFP-KRAS4b-Y71H	{Cirstea, 2013 #213}	N/A
Software and algorithms		
GuavaSoft version4.0	Cytek Biosciences	N/A
Leica Application Suite X version 4.12	Leica Microsystems	RRID: SCR_013673
GraphPad Prism version 9.5.1	GraphPad	RRID: SCR_002798
Fiji version 2.9.0	Schindelin, 2012	RRID: SCR_002285
FlowFate version 1.2	{Parisi, 2023 #232}	N/A
Image Studio software version 5.2	LI-COR Biosciences	N/A
Bio-Rad CFX Manager version 3.1	Bio-Rad	N/A
FlowJo version 10 software	FlowJo	N/A
Other		
Guava easyCyte 6HT 2L flow cytometer	Cytek Biosciences	Cat# 0500-4007
Odyssey CLx Infrared Imaging System	LI-COR Biosciences	Cat# LI9140-00
CFX-connect real-time PCR Detection System	Bio-Rad	Cat# 1855200
Dulbecco's Modified Eagle's Medium (DMEM)	ThermoFisher Scientific	Cat# 11965092
Fetal bovine serum (FBS)	ThermoFisher Scientific	Cat# 10270106
Horse serum (HS)	ThermoFisher Scientific	Cat# 16050130
L-glutamine (200 mM)	ThermoFisher Scientific	Cat# A2916801
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher Scientific	Cat# 10378016
Opti-MEM Reduced Serum Medium	ThermoFisher Scientific	Cat# 31985070
Hank's balanced salt solution (HBSS)	Lonza	Cat# BE10-547F
Paraformaldehyde 16 % (w/v)	Avantor	Cat# 30525-89-4
TWEEN 20	Merck	Cat# P9416
Triton X-100	Merck	Cat# T8787
BsrGI-HF (20,000 U/mL)	New England BioLabs	Cat# R3575S

Table S2. Qualitative changes of biochemical properties of oncogenic Ras mutants. Information on the biochemical properties was compiled from references given in the main text. *Unless otherwise annotated in the table.

Mutation	Disease	Frequency	Intrinsic GTP hydrolysis	NF1 GAP-mediated GTP hydrolysis*	SOS1-mediated nucleotide exchange	RBD binding affinity
K-Ras G13D	cancer	12.8 %	impaired	**normal**	**increased**	decreased
K-Ras G12V	cancer	23.1 %	impaired	**impaired**	normal	decreased
K-Ras Q61H	cancer	0.75 %	impaired	**impaired (p120gap)**	mild increase	mild increase (predicted)
N-Ras G12V	cancer	1.7 %	*impaired (predicted based on K-RasG12V)*	***impaired (predicted)***	*normal (predicted)*	*decreased (predicted)*
H-Ras G12V	cancer	10.2 %	*impaired (predicted based on K-RasG12V)*	***impaired*** *(predicted)*	*normal (predicted)*	*decreased (predicted)*
K-Ras G12C	cancer	11.5 %	reduced	**impaired (p120gap)**	normal	mild decrease
K-Ras G12D	cancer	34.1 %	reduced	**impaired**	normal	decreased

Table S3. Qualitative changes of biochemical properties of RASopathy K-Ras mutants. Information on the biochemical properties was compiled from references given in the main text.

Mutation	Disease	Frequency	Intrinsic GTP hydrolysis	NF1 GAP-mediated GTP hydrolysis*	SOS1-mediated nucleotide exchange	RBD binding affinity
K-Ras T58I	NS	/	reduced	**normal**	normal	decreased
K-Ras P34L	NS	/	normal	**impaired**	normal	strongly decreased
K-Ras Y71H	NS	/	impaired	**higher**	normal	increased
K-Ras D153V	NS	/	reduced	**normal**	normal	normal
K-Ras Q22E	CFCS	/	normal	**reduced**	increased	decreased
K-Ras V14I	NS	/	normal	**normal**	increased	decreased
K-Ras K5E	CS	/	*normal (predicted)*	***normal*** *(predicted)*	*normal (predicted)*	*normal (predicted)*
K-Ras P34R	NS & CFCS	/	normal	**impaired**	normal	strongly decreased
K-Ras K5N	CS	/	normal	**normal**	normal	normal

K-Ras G60R	CFCS	/	impaired	**impaired**	normal	strongly decreased